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STAIN TECHNOLOGY

JULY, 1931

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Editor and Business Manager
H. J. CONN
Soil Bacteriologist, N. Y. Agr. Exp. Station

Consulting Editors
CHEMISTRY: W. C. HOLMES Color Lab., U. S. Dept. of Agriculture
ZOOLOGY: S. I. KORNHAUSER School of Medicine, Univ. of Louisville
PATHOLOGY: F. B. MALLORY Boston City Hospital
BOTANY: W. R. TAYLOR Univ. of Michigan

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Address all correspondence regarding manuscripts for publication, proofs, etc. to H. J. Conn, Agr. Exp. Station, Geneva, N. Y. Articles to be accepted for publication must ordinarily deal with the nature or uses of biological stains. Articles dealing with microscopic technic in general, however, will be accepted in most cases, if they have a close bearing on staining procedures. Brief notes concerning new staining methods are especially solicited. It is understood that articles submitted for publication have not appeared previously elsewhere and that they will not be offered for simultaneous appearance in other publications without the consent of the editor of STAIN TECHNOLOGY.

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Articles over two printed pages in length (i. e. about 800 words) must be preceded by a brief summary (100–400 words) which can be used as an author's abstract by abstracting journals. This summary must therefore be complete in itself, and must contain all the important facts brought out in the paper.

All illustrations should accompany the manuscript and should be numbered with the legend for each written on separate paper and attached loosely to the illustration. They should always be referred to in the text. Line drawings must be made in India ink. Photographs for half-tones must be on paper giving black and white tones and should preferably have a glossy finish. *Colored illustrations accepted only at author's expense.*

Authors are especially urged to make all staining formulae definite and accurate. If known to the author, the dye content of the stain should be given and the exact dye used should be specified. If this information is not known, the name of the manufacturer should be stated and that of the stain itself exactly as worded on the label. Certified stains should be specified by certification number.

STAIN TECHNOLOGY

VOLUME VI JULY, 1931 NUMBER 3

PROGRESS IN THE STANDARDIZATION OF STAINS

ARMY SPECIFICATIONS FOR BIOLOGICAL STAINS

About two years ago announcement was made[1] that specifications of certain stains were being drawn up by a committee of the Army Medical Department, in coöperation with the Biological Stain Commission. At present some of these are ready for publication.

Specifications for eight stains, methyl orange, congo red, brilliant green, methyl violet, crystal violet, acid fuchsin, safranin O, and methylene blue, have been submitted by the Medical Department to the Stain Commission, approved by interested manufacturers, and have been adopted by the Medical Department for use in purchases of stains. They are to be submitted by the Medical Department to the Federal Specifications Board for acceptance as master specifications by the Government. Similar specifications for basic fuchsin and malachite green are in course of preparation.

Tests show that no stain samples approved in recent years by the Stain Commission will be excluded by these specifications. On the other hand, it is not proved that all stains conforming with these specifications will prove satisfactory in actual use. Accordingly the Commission will continue the same kind of tests in the future as in the past; but in the case of these eight stains will request the manufacturers to bear these specifications in mind when preparing any batch to submit for certification.

The specifications for these eight stains are as follows:

METHYL ORANGE, C. I. No. 142

1. (a) Methyl orange shall be the sodium salt of p-sulfbenzene-azo-dimethylaniline $C_{14}H_{14}N_3O_3SNa$.
   (b) Appearance—orange yellow powder.
   (c) It shall be soluble in water with a golden yellow color.
   (d) It shall be soluble in alcohol.
   (e) A magenta-red solution with a reddish-brown precipitate shall result on the addition of concentrated hydrochloric acid to an aqueous solution of the dye.
   (f) An orange-yellow, soluble, precipitate shall form on the addition of 10% aqueous NaOH to an aqueous solution of the dye.

[1] H. J. Conn. Stain Specifications. Stain Techn. 4, 1–2. 1929.

(g) It shall produce a brown solution on concentrated sulfuric acid changing to a magenta-red on dilution with water.

(h) The dye content shall be not less than 90%.

(i) A solution of the dye of 10 parts in the million of 50% ethyl alcohol acidulated with a few drops of hydrochloric acid must exhibit a sharp, clean cut absorption curve when examined with a spectrophotometer with a maximum at 510 mμ. There shall be no secondary maxima.

2. The dye content shall be determined as follows:

(a) One gram of the dye shall consume not less than 110.03 mil. of N/10 titanous chloride.

Congo Red, C. I. No. 370

1. (a) Congo Red shall be the sodium salt of diphenyl-disazo-bis-α-naphthylamine-4-sulfonic acid. $C_{32}H_{22}N_6O_6S_2Na_2$.

(b) Appearance—reddish brown powder.

(c) It shall be soluble in water with a reddish-brown solution.

(d) A blue precipitate shall form upon the addition of concentrated hydrochloric acid to an aqueous solution of the dye.

(e) The addition of 10% aqueous NaOH to an aqueous solution of the dye shall result in a reddish-brown precipitate, soluble in water.

(f) A bluish-violet precipitate shall form upon the addition of dilute acetic acid to an aqueous solution of the dye.

(g) A blue solution shall form upon concentrated sulfuric acid with a blue precipitate upon dilution with water.

(h) The dye content shall not be less than 80%.

2. The dye content shall be determined as follows:

(a) One gram of the dye shall consume not less than 91.90 mil. of N/10 titanous chloride.

Brilliant Green, C. I. No. 662

1. (a) Brilliant green shall be the sulfate of tetraethyl-diamino-tri-phenylcarbinol-anhydride. $C_{27}H_{33}N_2.SO_4H$. The oxalate salt is prohibited.

(b) Appearance—small glistening golden crystals.

(c) It shall be readily soluble in water with a green color.

(d) It shall be soluble in alcohol with a green color.

(e) A reddish-yellow solution shall result on the addition of concentrated hydrochloric acid to an aqueous solution of the dye.

(f) With the addition of 10% aqueous NaOH to an aqueous solution of the dye there shall be a pale green precipitate of the color base.

(g) A yellowish solution shall form on concentrated sulfuric acid changing thru reddish-yellow to yellowish-green and lastly to a green color on dilution with water.

(h) No precipitation shall occur upon the addition of a 10% aqueous solution of calcium chloride to an aqueous solution of the dye, until an excess of the CaCl has been added.

(i) The dye content shall not be less than 83%.

(j) A solution of the dye of 10 parts in the million of 50% ethyl alcohol must exhibit a sharp clean cut absorption curve when examined with the spectrophotometer with a maximum at 631 mμ. There shall be no secondary maxima.

2. The dye content shall be determined as follows:

(a) A solution of 10 parts in the million of 50% ethyl alcohol examined with the spectrophotometer in a 1.0 cm. cell shall show absorptive indices of not less than 1.03 at 620 mμ nor less than 1.13 at 630 mμ; or

(b) One gram of the dye shall consume not less than 34.41 mil. of N/10 titanous chloride.

Methyl Violet, C. I. No. 680

1. (a) Methyl violet shall be the chloride of the more highly methylated para-rosanilins, principally penta-methyl-para-rosanilin. For purposes of estimation the following formula will be applied: $C_{24}H_{28}N_3Cl$.

   (b) Appearance—green metallic glistening lumps or powder.

   (c) It shall be readily soluble in water with a violet color.

   (d) It shall be soluble in ethyl or amyl alcohol with a violet color.

   (e) The addition of concentrated hydrochloric acid to an aqueous solution of the dye shall result first in a blue solution, then a green solution and finally a yellowish-brown solution.

   (f) The addition of 10% aqueous NaOH to an aqueous solution of the dye shall result in a brownish-red coloration and precipitate.

   (g) It shall form a yellow solution on concentrated sulfuric acid changing to a yellowish-green, greenish-blue and then a violet solution on dilution with water.

   (h) The dye content shall not be less than 82%.

   (i) A solution of the dye of 10 parts in the million of 50% ethyl alcohol shall exhibit a sharp, clean cut absorption curve when examined with the spectrophotometer with a maximum at 580 mμ. There shall be no secondary maxima.

2. The dye content shall be determined as follows:

   (a) A solution of dye of 10 parts in the million of 50% ethyl alcohol examined with the spectrophotometer in a 1.0 cm. cell shall show absorptive indices of not less than 1.97 at 580 mμ nor less than 1.56 at 550 mμ; or

   (b) One gram of the dye shall consume not less than 41.66 mil. of N/10 titanous chloride.

Crystal Violet, C. I. No. 681

1. (a) Crystal violet shall be the chloride of hexa-methyl-para-rosanilin.

   (b) Appearance—bronzy glistening crystals or cantharides like glistening crystals or powder.

   (c) It shall be readily soluble in water with a violet solution.

   (d) It shall be soluble in alcohol with a violet color.

   (e) The addition of concentrated hydrochloric acid to an aqueous solution of the dye shall result successively in a blue, green and finally a yellow solution.

   (f) The addition of 10% aqueous NaOH to an aqueous solution of the dye shall result in a violet precipitate of the color base.

   (g) A yellow solution shall form on concentrated sulfuric acid which shall change to green, blue and then violet solution on dilution with water.

   (h) The dye content shall be not less than 85%.

   (i) A solution of the dye of 10 parts in the million of 10% acetic acid in water must exhibit a sharp clean cut absorption curve when examined with the spectrophotometer with a maximum at 591 mμ. There shall be no secondary maxima.

2. The dye content shall be determined as follows:

   (a) A solution of dye of 10 parts in the million of 10% acetic acid examined with the spectrophotometer in a 1.0 cm. cell shall show absorptive indices of not less than 2.12 at 590 mμ or of not less than 1.87 at 580 mμ; or

   (b) One gram of the dye shall consume 41.7 mil. of N/10 titanous chloride.

Acid Fuchsin, C. I. No. 692

1. (a) Acid fuchsin shall be the disodium salt of the di and tri sulfonic acids of rosanilin. It shall be estimated on the basis of $C_{20}H_{17}N_3O_9S_3Na_2$.

   (b) Appearance—glistening metallic-green granules or a dark red powder.

   (c) It shall be readily soluble in water with a bluish red solution.

   (d) It shall be almost insoluble in alcohol.

   (e) There shall be no change on the addition of concentrated hydrochloric acid to aqueous solution of the dye.

(f) An aqueous solution of the dye shall be almost completely decolorized by the addition of 10% sodium hydroxide but the color shall be restored by acids, even carbon dioxide.

(g) A yellow solution shall form on concentrated sulfuric acid which shall gradually change to red upon dilution with water.

(h) The dye content shall be not less than 60%.

(i) A solution of the dye of 10 parts in the million of 50% ethyl alcohol slightly acidulated with a few drops of hydrochloric acid, must exhibit a sharp, clean cut absorption curve when examined with the spectrophotometer with a maximum at 553 mμ. There shall be no secondary maxima.

2. The dye content shall be determined by either of the following methods:

(a) A solution of the dye of 10 parts in the million of acidulated 50% ethyl alcohol when examined with the spectrophotometer in a 1.0 cm. cell shall show absorptive indices of not less than 0.933 at 550 mμ or of 0.789 at 560 mμ.

(b) One gram of the dry dye shall consume 20.50 mil. of N/10 titanous chloride.

Safranin O, C. I. No. 841

1. (a) Safranin O shall be a mixture of diamino-phenyl-ditolazonium chloride and diamino-o-tolyl-ditolazonium-chloride. $C_{26}H_{19}N_4Cl$ and $C_{27}H_{21}N_4Cl$. Estimation will be made on the basis of equal parts of the two.

(b) Appearance—reddish brown powder.

(c) It shall be soluble in water with a red solution.

(d) It shall be soluble in alcohol with a red solution having a yellowish-red fluorescence.

(e) The addition of concentrated hydrochloric acid to an aqueous solution of the dye shall result in a bluish-violet solution.

(f) The addition of 10% aqueous NaOH to an aqueous solution of the dye shall result in a brownish-red precipitate.

(g) A green solution shall result on concentrated sulfuric acid which shall change to blue and then to a red solution on dilution with water.

(h) The dye content shall not be less than 85%.

(i) A solution of the dye of 10 parts in the million of 50% ethyl alcohol must exhibit a sharp clean cut absorption curve when examined with the spectrophotometer with a maximum at 530 mμ. There shall be no secondary maxima.

2. The dye content shall be determined as follows:

(a) A solution of dye of 10 parts in the million of 50 % ethyl alcohol examined with the spectrophotometer in a 1.0 cm. cell shall show absorptive indices of not less than 13.1 at 530 mμ nor less than 1.15 at 520 mμ; or

(b) One gram of dye shall consume not less than 47.55 mil. of N/10 titanous chloride.

Methylene Blue, C. I. No. 922

1. (a) Methylene blue shall be essentially tetramethyldiamino-diphenaxthionium chloride. $C_{16}H_{18}N_3SCl$. There shall be a slight admixture of lower homologs and oxidation products.

(b) Appearance—dark green powder with a metallic luster.

(c) It shall be readily soluble in water with a blue color.

(d) It shall be less readily soluble in alcohol than water.

(e) There shall be no change on the addition of concentrated hydrochloric acid to an aqueous solution of the dye.

(f) The addition of 10% aqueous sodium hydroxide shall first result in a more violet solution followed by a dull violet precipitate in an aqueous solution of the dye.

(g) A yellowish-green solution shall result on concentrated sulfuric acid which shall change to a blue solution upon dilution with water.

(h) The dye content shall be not less than 85%.

(i) A solution of the dye of 10 parts in the million of 50% ethyl alcohol shall exhibit a sharp, clean cut absorption curve when examined with the spectrophotometer with a maximum absorption at about 665 mμ. There shall be a slight secondary maximum in the vicinity of 610–615 mμ, due to oxidation products.

2. The dye content shall be determined as follows:

(a) A solution of dye of 10 parts in the million of 50% ethyl alcohol examined with the spectrophotometer in a 1.0 cm. cell shall show absorptive indices of not less than 2.21 at 660 mμ and of not less than 1.05 at 610 mμ; or

(b) One gram of the dry dye shall consume not less than 53.18 mil. of N/10 titanous chloride.

THE ATMOSPHERIC DEALKYLATION OF AQUEOUS SOLUTIONS OF CRESYL BLUE

W. C. HOLMES[1] AND A. R. PETERSON.[2]

Contribution No. 193 from the Color and Farm Waste Division, Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington, D. C.

ABSTRACT.—**Aqueous solutions of cresyl blue are decidedly less stable than those of methylene blue. An appreciable degree of dealkylation occurs even under only slightly alkaline conditions (e. g. pH 7.68) and at room temperatures within a period of a few weeks. At pH 9 the dye is distinctly affected within twenty-four hours.**

The writers recently had occasion to examine two dilute (0.1%) solutions of cresyl blue in distilled water which had been prepared more than a year before and set aside. The dye in question is dimethyldiaminophenotolazoxonium chloride (C. I. No. 887), prepared by the condensation of nitroso-m-dimethylamino-p-cresol and p-phenylenediamine. The maximum of its absorption band in aqueous solution is approximately 625mμ. The absorption of the aged solutions in question proved very different. Their bands were found to have shifted about 17.5 mμ toward the violet, so that their maxima fell at approximately 607.5 mμ.

The dye in these solutions was free from oxazones and retained the characteristic properties of oxazine dyes, so that the possibility of any fundamental modification of the cresyl blue molecule appears precluded. It seems evident that the alteration is a dealkylation of the same general type that occurs under similar conditions with methylene blue.[3]

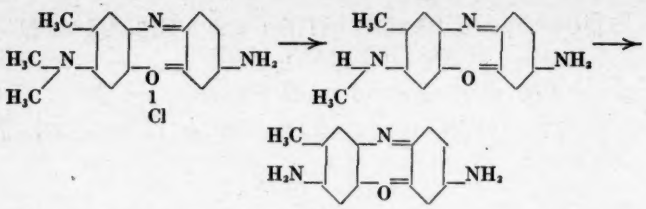
It was found possible to duplicate the color content in these aged solutions very closely by boiling dilute acid solutions of cresyl blue with small concentrations of potassium bichromate for a brief period.

When cresyl blue is treated in this manner with increasing proportions of bichromate the absorption band shifts progressively toward the violet until its maximum falls at approximately 595 mμ, beyond which point no further alteration seems to occur. The ultimate product thus obtained appears identical with diaminophenotolazoxonium chloride.

[1]Senior Chemist, Color and Farm Waste Division, Bureau of Chemistry and Soils, Washington, D. C.

[2]Research Associate, Commission on Standardization of Biological Stains.

[3]Holmes, W. C., and French, R. W. The oxidation products of methylene blue. Stain Techn., 1, 17. 1926.



The degree of dealkylation which cresyl blue has undergone in any instance is indicated by the location of its absorption maximum within the spectral range, 625 to 595 mμ.

In view of the importance of cresyl blue in vital staining and other biological applications, tests were undertaken to define more closely the conditions under which its aqueous solutions prove unstable.

The effect of decidedly alkaline conditions was investigated first. Dilute solutions of the dye were made up with buffer provisions, from which aliquots were removed from time to time for examination. The general provisions of the tests were identical with those previously employed in a similar investigation of methylene blue,[4] to which the reader is referred for detailed description. The only modification introduced was the adoption of a different spectrophotometric ratio for the evaluation of the aged solutions. The ratio of the extinction coefficients of the solutions at 605 mμ to their extinction coefficients at 635 mμ was selected as being suitable with cresyl blue.

TABLE 1. STABILITY OF CRESYL BLUE UNDER ALKALINE CONDITIONS

(R = E at 605 mμ ÷ E at 635 mμ)

Time	Distilled Water	pH 8.98	pH 9.76	pH 10.87	pH 11.43
	(R)	(R)	(R)	(R)	(R)
Immediate	0.88	0.88	0.87	0.88	0.89
1 day	0.87	0.97	1.08	1.11	(1.44)
2 days	0.88	1.10	1.39	—	—
4 days	0.88*	1.41	—	—	—

*The ratio (R) of this solution was found to be 0.93 when examined about five weeks later.

With the dye sample employed in these tests this ratio (R) was found to have the value of 0.88 (±0.02). It will be evident that this value will increase as the absorption band shifts toward the violet and that the increment will parallel increasing dealkylation in the dye. A spectrophotometric ratio of this type affords a much more

[4]Holmes, W. C., and Snyder, E. F. The atmospheric oxidation, or dealkylation, of aqueous solutions of methylene blue. Stain Techn., 4, 7. 1928.

reliable and accurate means of detecting alteration in dyes than does the determination of the locations of absorption maxima. The ratio value of the aged stock solutions referred to in the opening paragraph of this paper (which were prepared from the same dye sample as that used in these tests) was found to be 1.33.

The influence of alkalinity upon the stability of the dye solutions is shown in Table 1.

With increasing alkalinity, aqueous solutions of cresyl blue undergo decolorization with increasing rapidity. This phenomenon rendered protracted observations on the solutions impossible but did not other-

TABLE 2. STABILITY OF CRESYL BLUE NEAR NEUTRALITY

(R = E at 605 mμ ÷ E at 635 mμ)

Time	pH 6.64	pH 7.08	pH 7.68
	(R)	(R)	(R)
Immediate	0.88	0.88	0.89
1 week	0.88	0.90	0.91
2 weeks	0.89	0.90	0.94
3 weeks	0.88	0.92	0.96
4 weeks	0.90	0.93	0.99

wise interfere with the tests. It is evident that cresyl blue undergoes comparatively rapid dealkylation under alkaline conditions. It is far less stable under such conditions than is methylene blue. Cresyl blue appears to undergo in one day at pH 9.0 a degree of dealkylation which is about the same as that which methylene blue undergoes in four weeks at pH 10.0.

The effects under alkaline conditions were so extreme that a second series of tests was carried out under slightly alkaline, approximately neutral and slightly acid conditions. The results obtained are recorded in Table 2.

Assuming, as appears justified, that 0.03 in ratio value is an ample allowance for possible experimental inaccuracy, it may be concluded that it is questionable if dealkylation occurs at pH 6.64 over a period of a month, but that it does occur in a slight degree at pH 7.08. At pH 7.68 dealkylation is appreciable.

It appears probable that such quinonimide dyes as are alkylamino derivatives are, in general, susceptible in varying degrees to dealkylation of this type. Aqueous stock solutions of such dyes may undergo dealkylation on standing, particularly if kept in glass containers which give up alkali at all readily. Dealkylation may occur rapidly

under decidedly alkaline conditions. Such alterations must necessarily modify the behavior of the dyes in biological applications.

It would appear, upon the other hand, that alkylamino derivatives in the triphenylmethane series do not undergo dealkylation readily, if at all. Crystal violet, for example, has been investigated in this laboratory and found to be stable under relatively drastic treatment.

NEW RAPID METHODS FOR TISSUE DIAGNOSIS

AXEL M. HJORT AND CARL H. MOULTON
Med. Research Div., Chemical Warfare Service, Edgewood Arsenal, Md.

ABSTRACT.—**Two new methods applicable to the staining of fixed and fresh frozen tissue sections are presented herein. In addition certain improvements are suggested for the technic reported by Geschickter, Walker, Hjort and Moulton (1931). In brief the procedures are as follows:**

I. The thionin eosinate method of Geschickter *et al* (1931). This procedure has been modified as follows:

1. A mixture of diethylene glycol, 40 parts, ethylene glycol, 40 parts, and grain alcohol, 20 parts is superior to ethylene glycol, 80 parts, and ethyl alcohol, 20 parts, as a solvent for the compound stain in that the staining is intensified.

2. Ethylene glycol monobutyl ether supplants diethylene glycol monobutyl ether because of its lower viscosity.

3. Ethyl phthalate replaces butyl phthalate on account of a more satisfactory viscosity.

II. The methyl green eosinate procedure is the same as the modified thionin eosinate method except for the following variations:

1. The staining time is increased to one minute.

2. Decolorization and washing are reduced to about 15 seconds.

III. The hematoxylin-eosin method. After cutting, the tissue sections are carried thru the following steps:

Unfold in water; transfer to formalin (4 to 40%) for at least 30 seconds; stain in hematoxylin (Harris) for 30 to 60 seconds; wash in water, 5 seconds; decolorize in 0.1% HCl or saturated aqueous picric acid, 5 seconds; wash in water, 5 seconds; float in 0.5% ammonia, 5 to 10 seconds; wash in water, 5 seconds; stain in 5% aqueous eosin, 15 seconds[1]; wash in water, 5 to 10 seconds; dehydrate in a mixture of diethylene glycol, 30 parts, and ethyl alcohol, 70 parts, 5 to 10 seconds; dehydrate in ethylene glycol monobutyl ether, 5 to 10 seconds; clear in ethyl phthalate, 5 to 10 seconds; float on a glass slide, blot with photographic lintless blotter, place a drop of neutral gum damar on the section, and cover with glass cover slip.

INTRODUCTION

Considerable progress has been made in the recognition of malignant growths, but improvement in rapid staining methods for facilitat-

[1]See variant in detailed account in text.

ing tissue diagnosis has not kept apace. Save for a few recent developments in stain technology, polychrome methylene blue is still almost universally used where speed is necessary. Polychrome methylene blue, tho valuable in the absence of a better stain, is not all that is desired. This stain, in the hands of some technicians, is, no doubt, a good diagnostic instrument, but where a limited amount of material is studied and funds are inadequate, polychrome methylene blue is found wanting both in its differential qualities and in permanency. Any method, which does not appreciably affect speed, but will improve differentiation, and attain permanency, should prove a distinct asset to the average technician. Some progress has recently been made in this direction.

Differentiation may be attained by either of two methods, the first of which, consists of staining and counterstaining as is practiced in the ordinary hematoxylin-eosin procedures, and the second, is performed thru the agency of compound stains such as the Ehrlich, Romanovsky, Nocht, Leishman, Wright, Giemsa and MacNeal stains. The compound stains have been recommended primarily for blood staining, hence it is apparent that more progress has been made in the evolution of blood stains than in stains for other tissues.

Within the past year several new methods for the rapid staining of tissue have been recommended. There are three, in particular, which employ compound stains.

1. Rhamy (1930) suggests the use of a basic fuchsin, eosin and methylene blue mixture dissolved in approximately 40% grain alcohol. The cut frozen sections are prepared for staining by treatment with 4% formalin, and after staining are washed in water, dehydrated in 96% grain alcohol and absolute grain alcohol, cleared in xylol, and mounted in xylol-balsam. In our experience, this method is deficient in differential staining qualities, and the alcohol and xylol treatment causes too much hardening and shrinking. If the tissue is previously immersed in 4 to 40 % formalin for hours or days, the undesirable alcohol and xylol effects are not encountered.

2. Compound dyes, with few exceptions, are relatively insoluble in water and sparingly soluble in alcohol. Geschickter (1930), however, came upon one exception in the water soluble compound of azure A and Erie garnet B. This stain is a distinct improvement on the polychrome methylene blue procedure, but still remains a water technic, and thus, is lacking in permanency.

3. Grain alcohol is one of the most commonly used organic solvents in many industries. It possesses many desirable qualities for numerous purposes but is most unsuitable for many biological uses,

because of its limited solvent action and its precipitating effect on protein. One of us (A. M. H.) has been conducting an investigation on the application of a series of closely allied organic solvents where alcohol cannot be employed. As stated above, compound dyes are sparingly soluble in alcohol, but many of them are quite soluble in at least one of the solvents investigated, namely, ethylene glycol. This property of ethylene glycol has proved valuable in the development of another rapid staining procedure by Geschickter, Hjort, Walker and Moulton (1931). For a detailed account of this method, reading of the original article is recommended.

The present authors have made certain modifications in the last mentioned method, and have added another compound stain for employment with the same general procedure. In addition, they have modified a hematoxylin-eosin method for application to the rapid staining of fresh frozen tissue.

General Considerations

Any ideal rapid staining procedure must possess the following fundamental characteristics: 1) High degree of differentiation; 2) Rapid staining (the whole procedure should not take more than 5 minutes); 3) The solvents employed should have a minimum skrinking and hardening effect on the tissue; 4) Uniformity of staining; 5) Permanency.

The method of Geschickter, Hjort *et al* (1931) has all of these save the last. Some specimens have retained the stain for 5 to 6 months while others fade within a day to a week. Attempts by the present authors to prevent fading in this particular technic have so far proved unavailing. More complete dehydration, hardening with formalin, and destruction of ferments by means of potassium cyanide and sodium fluoride have been tried but the results have been disappointing. Likewise, the use of buffers of pH 7.0 have proved unsuccessful. One guess is as good as another, at the present time, in explaining the cause of the fading.

Precision in technic is essential in some of the steps of the Geschickter, Hjort *et al* procedure. These are carefully described in the original article, but some points require emphasis. In the first place, a sharp microtome knife is paramount for frozen section work. The Fanz automatic microtome knife sharpener, or its equivalent, is almost indispensable. The thinner the sections the more satisfactory they are for diagnosis, but for the beginner sections of 10 to 20 μ thickness are recommended. To obtain the desired thinness of sections without tearing and curling, complete embedding in ice and cutting

with a slow stroke of the knife across the block is advised. Some practice is necessary before satisfactory frozen sections can be made with uniformity. In this instance, the art of the technic enters. If the frozen block is too hard, the cut sections crumble, and if too soft, the knife either does not cut at all or only irregularly. Practice alone teaches when to cut. All sections should be unfurled in the water collecting bath. If not unfurled here, they usually remain folded thruout the procedure. Uniformity of staining is facilitated by floating the sections free from the glass rod in each bath.

The compound stains examined were mostly combinations of eosin, an acid dye, with numerous basic dyes. Combinations of blue and red, and yellow, blue, and red have been given quite extensive consideration, but so far only thionin eosinate and methyl green eosinate have proved serviceable for rapid staining. In the case of the others, either the stains do not penetrate the cells sufficiently or they are too soluble in the solvents employed in washing, dehydrating and clearing. There are, no doubt, other compound stains which will prove serviceable in the present technic or its modifications.

The solvents employed must possess certain properties, as follows: 1) They must dissolve the stain in adequate concentration for tissue staining. 2) They must have little if any shrinking and hardening action on the cut sections. 3) They must serve to wash, dehydrate and clear the stained sections.

A series of solvents which fulfil the above requirements has been found. For those unacquainted with the terminology and nature of these solvents a brief description follows:

1. Ethylene glycol ($HOCH_2CH_2OH$), the commercial antifreeze, "prestone".[2]

2. Diethylene glycol ($HOCH_2CH_2OCH_2CH_2OH$), or dihydroxyethyl ether.

3. Ethylene glycol monobutyl ether ($HOCH_2CH_2OCH_2CH_2CH_2CH_3$).

4. Diethylene glycol monobutyl ether ($HOCH_2CH_2OCH_2CH_2OCH_2CH_2CH_2CH_3$).

5. Ethyl phthalate [$C_6H_4(CO_2C_2H_5)_2$].

6. Butyl phthalate [$C_6H_4(CO_2C_4H_9)_2$].

Other solvents have been employed with more or less success, but we believe the above are the best at the present state of our knowledge.

It will be noted that alcohol is mixed with ethylene glycol, diethylene glycol and with glycerin in certain steps of the original and modi-

[2]Do not purchase commercial prestone, however, for it now contains a blue coloring matter.

fied procedure. The alcohol serves the purpose of reducing the viscosity of these compounds, and in turn they prevent shrinking and hardening effect of the alcohol.

These solvents cover quite a range of solubility. There is a definite reason for the employment of each solvent mixture or simple solvent at the various steps in the procedure. Ethylene glycol plus alcohol, and a mixture of ethylene glycol, diethylene glycol, and alcohol are the best stain solvents. The limited solubility of the stains in the washing baths favors their retention in the tissues. Ethylene glycol and diethylene glycol are not very soluble in ethyl or butyl phthalate, hence, an intermediate bath, which dissolves the former two and is soluble in the latter two, is essential. Such solvents as ethylene glycol monobutyl ether and diethylene glycol monobutyl ether are employed for this bath. There are other solvents which fulfil the solvent requirements, but are either too viscid or not viscid enough.

The ordinary hematoxylin-eosin procedures are not directly applicable to the staining of fresh frozen tissue because they require too much time and contain solvents which markedly shrink and harden such tissue. By making certain modifications in this technic, we have found it quite adaptable to a rapid staining procedure for fresh tissue. Employment of this technic has resulted in a stain which fulfils every requirement outlined at the beginning of this section, page 85.

New Methods

Compound Dyes

1. Thionin eosinate (*a modification of the method of Geschickter, Hjort et al 1931*). A few changes in this technic have suggested the following outline:

a. Freeze the tissue block.

b. Cut the sections 5 to 10μ in thickness. Wet the finger and transfer the sections from the knife to a water bath where they are unfolded.

c. Transfer the sections by means of a glass rod to the phosphate buffer solution of pH 7.0 and all subsequent baths. Float in the buffer for at least 15 seconds.

d. Float on a dehydrating bath consisting of diethylene glycol, 30 parts, and 95% grain alcohol, 70 parts for 5 seconds.

e. Float in the compound stain for 10 to 15 seconds. This stain may be of either of the following formulae:[3]

1. Thionin eosinate 1%.

2. Thionin eosinate 1% and the barium or calcium salts of eosin or erythrosin, 0.5%.

3. Thionin eosinate 0.8 to 1.0%, and eosin (color acid) 0.2%.

The first two are preferable.

f. Float for 5 to 10 seconds in the first of two washing baths of the same composition as the dehydrating bath in (d). Then float and stir about in the second washing bath for 30 to 60 seconds or until the desired color is obtained. Practice is necessary to do this consistently. If the red element is deficient, the section is returned to the staining bath for 5 seconds, where now, due to the dehydrated condition of the tissue, only the acid stain enters. Repeat the treatment in the washing baths, after which pass on to the subsequent steps.

g. Pass the section into ethylene glycol monobutyl ether for 5 seconds. This solvent has replaced diethylene glycol monobutyl ether because of its lower viscosity. A second bath of the same solvent may be introduced, if desired.

h. Float onto a bath consisting of ethyl phthalate for 5 to 10 seconds. Here, too, a second bath of the same solvent may be employed. Ethyl phthalate has supplanted butyl phthalate because it is less oily and, therefore, blots more readily.

i. Float the section on a glass slide, blot with bibulous paper, or lintless blotter, and mount with *neutral* xylol-gum-damar.

The total time required for this method is between 3 and 5 minutes. Delicate tissues like the lung, liver and intestine should be handled with great care.

2. Methyl green eosinate. This dye is dissolved in the same solvent employed in making up the thionin eosinate preparations. It may be used in 1% solution alone, or in 0.8% solution with 0.2% eosin (color acid). The technic is essentially the same as employed for thionin eosinate, with the following exceptions:

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[3]The solvent employed for these dyes is a mixture of diethylene glycol, 40 parts, ethylene glycol, 40 parts, and 95% grain alcohol, 20 parts. Diethylene glycol intensifies the stain, but the dyes are not sufficiently soluble in it, hence the mixture is recommended. The Abbott Laboratories of North Chicago, Illinois, will make the compound dyes, staining solutions and solvents available. For those who wish to prepare the stains themselves it is suggested that the dyes be ground with the ethylene glycol in a glass mortar and then the diethylene glycol and alcohol are added with constant stirring and grinding followed by filtration thru paper.

a. The tissue sections are floated on the stain for 60 seconds instead of 15.

b. Washing in the wash baths is shorter to prevent too much decolorization, and counterstaining is unnecessary. The increased time of staining is balanced by the decreased time of washing, thus making the length of the two methods the same.

Methyl green eosinate is included because it may prove valuable for certain purposes. Tissues stained by this dye retain their color about as well as those stained by means of thionin eosinate, but permanency in either case is not assured at the present time.

3. Other Eosinates. The eosinates of azure A, toluidine blue, crystal violet and a number of other basic blue and yellow dyes have been tried but have not proven satisfactory with the present technic. Just what part the observations of Irwin (1930) on the penetrability of nuclear stains play in this work is not known.

STAINING AND COUNTERSTAINING WITH SIMPLE STAINS

In the section on general considerations, it was stated that the ordinary hematoxylin-eosin procedures could not be employed unmodified in frozen section work. By choosing the proper hematoxylin stain, however, and modifying the dehydrating and clearing procedure, hematoxylin staining with eosin counterstaining may be employed in frozen section work without appreciably increasing the time required. This method has the advantage of yielding permanency of color in the stained materials. No fading has taken place in six weeks, the time when the first sections were stained.

Two preparations of hematoxylin have been tried, namely, the Delafield and the Harris formulae. The latter is the better of the two and is employed in the method outlined below:

a. The fresh tissue sections are cut and then collected and unfolded in water. They are transferred to a formalin solution of 4 to 40% strength. They may remain in this solution indefinitely but at least 15 seconds is recommended. Little if any gross shrinkage occurs.

b. A glass rod is employed for transferring the sections from the formalin to the hematoxylin (Harris) stain and all subsequent steps. Stain for 30 to 60 seconds.

c. Float into water for 5 seconds.

d. Decolorize in 0.1% hydrochloric acid for 5 seconds, or in a saturated aqueous picric acid solution for 5 to 10 seconds.

e. Transfer to water for 5 seconds.

f. Float onto 0.5% ammonia for 5 to 10 seconds.

g. Wash in water for 5 seconds.

h. Pass into a mixture of diethylene glycol, 30 parts, and 95% grain alcohol, 70 parts, for 5 seconds to prepare for counterstaining in eosin.

i. Float onto eosin counterstain for one minute. This stain is a 1% solution of eosin (color acid) in a mixture of diethylene glycol, 40 parts, ethylene glycol, 40 parts, and 95% grain alcohol, 20 parts.

j. Pass for 5 to 10 seconds into a dehydrating bath of the same composition as (h) above, but which is saturated with calcium hydroxide.[4] The purpose of the calcium hydroxide is to make the eosin less soluble by the formation of the calcium salt.

k. Float onto another dehydrating bath of the same composition as (h) for 5 seconds.

l. Transfer to ethylene glycol monobutyl ether for 5 seconds. A second bath of this material may be used as in the case of the compound stain technic.

m. Float onto ethyl phthalate for 5 seconds. Again a second bath of this material may be employed.

n. Float on a glass slide, blot with bibulous paper or with a lintless blotter and mount in neutral gum damar or balsam. This procedure requires about 5 minutes for completion.

A variant of this method which saves some time is to omit (h), substitute 5% aqueous eosin for the 1% eosin (color acid) wherein the section is stained for 15 seconds, omit (j) and substitute for it a water rinse followed by two successive baths of the same composition as (h), after which the procedure remains as above.

The hematoxylin-eosin technic may also be carried out on sections which are fixed on a glass slide by means of an egg albumen-glycerin film before passing into the formalin. This is recommended for delicate tissues like the lung, intestine, liver, etc.

Material which has been fixed for some hours or days in formalin may be stained by the ordinary hematoxylin-eosin procedure for then the alcohol and xylol do not cause much shrinkage.

The modified technic outlined above may also be used in staining formalin fixed, paraffin blocked, and pyroxylin blocked tissue. In the case of paraffin and pyroxylin fixed tissue, the same precautions must be observed as in the ordinary technic, and such material requires longer staining in hematoxylin.

MATERIALS AND APPARATUS

These are recorded in detail in the article by Geschickter, Hjort *et al* (1931), and need not be repeated here.

[4]Add an excess of calcium hydroxide to the solvent; shake well; let stand until clear; and finally decant the clear fluid for use in this step of the procedure.

Acknowledgments

The authors wish to express their appreciation to Dr. C. F. Geschickter of the Surgical Pathology Laboratory and Dr. J. E. Kemp of the Dermatological Service of Johns Hopkins Medical School for providing the specimens of human tissues employed in this investigation.

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A Correction

In the number of Stain Technology, Vol. 5, No. 3, July, 1930 and in Vol. 6, No. 1, January, 1931, there were reported from the Surgical Pathological Laboratory at the Johns Hopkins Hospital, two staining technics, both adapted to frozen sections, one employing mixtures of Erie garnet and azure A, best suited to rapid sections on fresh unfixed material; the other employing thionin eosinate, barium eosinate and azure A. We wish to correct now an omission and make an acknowledgment in regard to the dyestuffs employed in both of these technics.

In regard to the aqueous mixture of azure A and Erie garnet, both of these dry dyes can be obtained from the National Aniline and Chemical Company, 40 Rector Street, New York City, who were kind enough to furnish us with the initial samples during the progress of the investigation. In regard to the other staining technic, the compounds from which thionin eosinate and barium eosinate can be prepared can be obtained from the same company.

Those who wish to check the experiments and technics of these papers may secure these materials from National Aniline and Chemical Company as stated.—C. F. Geschickter

ISOHEMATEIN AS A BIOLOGICAL STAIN

ELBERT C. COLE

Williams College[1] *Williamstown, Mass.*

ABSTRACT.—**The possible use of isohematein as a biological stain is considered. Certain characteristics of the dye are discussed in relation to staining technic. A preliminary series of experiments is described. The stomach of the frog, skeletal muscle of the frog, and spinal cord of the cat were used as representative tissues. The dye has greater tinctorial power than hematoxylin (hematein) but it is not so selective for nuclei. The results at hand indicate that the dye may have some value as a differential stain for nerve cell bodies. Fibrillae in smooth muscle cells and cross striations in skeletal muscle were also brought out.**

Isohematein is said to have a greater tinctorial power than hematein.[2] So far as the writer is able to discover its possible value as a biological stain has not hitherto been reported.[3] The MacAndrews and Forbes Company kindly consented to prepare a sample lot.[4] This paper reports preliminary experiments made by the writer with a view to determining whether or not this dye is of value as a biological stain.

GENERAL CHARACTERISTICS OF THE DYE.

1. Isohematein is in the form of small jet black glossy crystals.
2. It is soluble in ethyl alcohol of 70% or higher grades. In solutions below 70% there is a general precipitation. It appears to be insoluble in water.
3. The dye requires a mordant for successful staining of animal tissues. In this respect it resembles hematoxylin.
4. The color of a 1% alcoholic solution is a deep brown with a suggestion of purple. The addition of a trace of ammonia increases the purple component of the color. The addition of small quantities of HCl has little if any effect upon the color of the dye solution.

[1]This investigation was carried on at the University of Arizona. The writer wishes to express to that institution and to Dr. Horace Gunthorp, Head of the Department of Biology, sincere appreciation for courtesies extended.

[2]Thorpe, Sir Edward. A Dictionary of Applied Chemistry, **14**, 152–159. 1922.

[3]Dr. H. J. Conn, in a personal communication dated August 5, 1930, informs me that he has not been able to find this dye listed in any dye index or stain catalog.

[4]The writer offers his thanks to Dr. H. J. Conn, for his interest in this matter and for his willingness to approach the MacAndrews and Forbes Company in an effort to secure a trial sample of this dye.

5. To test the keeping qualities of stock solutions two equal quantities of 2% isohematein in absolute ethyl alcohol were made up, one of them protected against oxidation by the addition of a trace of potassium meta-bisulfite. The bottles containing the solutions were stoppered, but there was a considerable amount of air above the surface of each solution. Four weeks later no difference in color could be noted. Tests showed very little if any difference in staining power of the two solutions.

Materials and Methods

Stock dye solution, 2% isohematein in absolute ethyl alcohol.

Ready stain, stock solution diluted 1:19 or 1:39 with 70% ethyl alcohol.

Mordants, 3% aqueous solutions of iron alum, ferric chloride or ferric acetate.

Tissues: skeletal muscles of frog, longitudinal sections 8 micra thick; pyloric stomach of frog, transverse sections 8 micra thick; spinal cord of cat, transverse sections 10 micra thick. Each of the tissues had been fixed in Bouin's picro-formol-acetic.

Sections, mounted on slides in the usual way, were passed thru xylol and down the alcohol series to the 70% grade. Slides were placed on a horizontal staining board and mordant applied with a pipette. After 15 minutes slides were rinsed in 50% alcohol and a quantity of ready stain flooded over them. Staining proceeded rapidly. The addition of a trace of ammonia to the ready stain was found to speed up the rate of staining greatly without apparent injury to the tissues. In from five to fifteen minutes the tissues were heavily stained. Slides were then rinsed and placed in a differentiating solution. The stain in the tissues was scarcely affected by HCl even when 1% solutions were used, but was very sensitive to 0.2% ferric chloride solution. There was a fair degree of differentiation.

Results

In sections of frog stomach mordanted with ferric chloride and stained with isohematein, the density was great but the contrast not very high, since the cytoplasm retained a considerable amount of the dye. The following color range in the cytoplasm is typical of slides which were prepared without any attempts at destaining:

Longitudinal muscle	purple-black to black
Circular muscle	purple-brown
Submucosa	light brown
Muscularis mucosae	purple-black to black
Mucosa, in general	dark brown

The entire coloration of such preparations was suggestive of the results frequently obtained with silver impregnation methods. Nuclei were heavily stained, usually black, but were more or less obscured by cytoplasmic staining. Results were practically identical regardless of which of the three mordants was used. Differences in the concentration of the ready stain did not affect the final picture, but, as might be expected, did affect the time required for the stain to reach a given density.

Some preparations were treated with destaining solutions to see if the contrast between nuclei and cytoplasm would be increased. Destaining proceeded very slowly in 0.8% HCl, but more rapidly in 0.2% iron alum and 0.2% ferric chloride. In any case the contrast secured was only fair. In preparations destained with ferric chloride solution, nuceli were black and cytoplasm varying shades of brown. Ganglion cells in the myenteric plexus, however, were stained a purple-black, in rather sharp contrast to the color of surrounding tissues. The fibrillar nature of smooth muscle cytoplasm was well marked.

In sections of the skeletal muscles of the frog, mordanted with ferric chloride, stained with isohematein and destained with ferric chloride, the nuclei were scarcely discernible. The cytoplasm was a light gray brown with cross striations clearly marked.

In sections of cat spinal cord, treated as above, the gray matter of the cord was stained a dense gray brown. The cell bodies of nerve cells were stained purple-black. These structures retained their color after the surrounding gray matter had become light brown as a result of further treatment with ferric chloride solution. The white matter of the cord changed in color from black to brown as a result of such treatment.

Similar preparations stained with hematoxylin were made for the purposes of comparison.

Conclusions

1. Isohematein has a greater tinctorial power than hematoxylin (hematein).

2. It is not so selective for nuclei, as nuclei are not so sharply differentiated from cytoplasm even after treatment with the usual destaining fluids.

3. It may have some value for preparations where gross delineation of regions is desired, different tissues tending to stain different colors ranging from light brown to purple-black.

4. It appears to act as a differential stain for nerve cell bodies, but its value for this purpose cannot be definitely established without further investigation.

5. Fibrillae in smooth muscle cells and cross striations in skeletal muscles are brought out by this dye.

6. In general, isohematein does not promise to excel hematoxylin in routine technic. Its value, if any, lies in its ability to stain nerve cell bodies selectively and to delineate fibrillae and cross striations in muscle cells.

A MODIFICATION OF MALLORY'S TRIPLE STAIN.

BORIS KRICHESKY, *Assoc. in Biology, Univ. of California at Los Angeles.*

Mallory's triple connective tissue stain, if successful, is excellent, tho somewhat uncertain and too frequently undependable even in the hands of the expert. The modification used by the author has given consistently good results when used on the hypophysis and has been used by others equally successfully on various other histological preparations. Differentiation is sharp, clear, and above all, consistent.

The method used consists not only in a modification of the concentration of the dyes of the stain but also in a modification of the procedure employed. For example, a weaker solution of acid fuchsin is used than that given in the popular texts on microtechnic. The concentration of anilin blue is increased and that of orange G is reduced. The modification in the procedure lies in the time required for washing and dehydrating.

Tissues fixed in Bouin's or Zenker's fixing fluids have given good results when used with this modification which is as follows:

I. Staining solutions:

Solution 1.

Acid fuchsin[1] (dye content 62%)	0.25 g.
Water	100 cc.

Solution 2.

A. Anilin blue[1]	2.0 g.
Water	100 cc.
B. Orange G[1] (dye content 83%)	1.0 g.
Water	100 cc.
C. Phosphomolybdic acid 1%	100 cc.

Solutions A, B, and C should be kept in separate bottles because the mixture deteriorates on standing. When ready for use, solution 2 is made up of equal parts A, B, and C.

II. Procedure:

If sections are 10 microns or less in thickness, hydrate by passing thru the descending series of alcohols to 70% alcohol and then directly into water for 5 minutes. Proceed with the steps as follows:

1. Transfer sections to solution 1 for from 1 to 3 minutes, the time to be estimated by experience.

[1]These three stains were all certified by the Stain Commission, and bore the certification numbers NR 2, NK 1, and NO 2, respectively.

2. Wash, by dipping the slide into water until the surface stain is removed.

3. Transfer to solution 2 for from 3 to 5 minutes or longer.

4. Wash, by dipping the slide into water from 1 to 3 times *only*.

5. Transfer the slide to 70% alcohol, dipping hurriedly about 3 times.

6. Transfer to 80% and then to 95% alcohol dipping the slide into each 5 to 8 times.

7. Dehydrate in absolute alcohol for several minutes.

8. Clear in xylol and mount.

It has been found that 2 minutes is the shortest time that sections can be immersed in solution 2 for good results. If a two minute immersion is used, eliminate step 4 and proceed directly with step 5. If sections are left for a longer time in solution 2, immersion in water is necessary (step 4). Since the anilin blue is readily soluble in water, the depth of this stain can be controlled by the amount of washing. All stains used were made by the National Aniline & Chemical Co., New York.

When the above method is used on histological preparations the remarkable differential qualities of the stain are brought out. Tho the procedure seems in part to be crude technic, excellent results have been obtained with it on sections of salivary glands, tongue, intestine, ovaries and other material. But the results obtained on sections of the hypophysis are exceptional. In the pars anterior, the nuclei of all cells are stained red, the cytoplasm of the eosinophil cells red, of the basophil cells blue, of the neutrophil cells orange, and of a group of cells which are believed to be the "indifferent" cells a gray color. In the pars intermedia the cytoplasm of the secreting cells is stained a pale blue and the colloid particles a bright red. The finely branched cells of the pars nervosa are stained a light blue as are also the small globules of colloid matter contained in it.

A DIFFERENTIAL STAIN FAVORABLE TO THE DIAGNOSIS OF NEISSERIAN INFECTION

SARA ALICIA SCUDDER,[1] *Bacteriologist, Department of Pathology, City Hospital, Welfare Island, New York City.*

ABSTRACT.—**A differential Gram stain has been evolved which incorporates the combined features of the original Gram and Pappenheim methods. National Aniline crystal violet and new methyl green and pyronin are the dyes preferred. The iodine mixture of Kopeloff and Beerman is a satisfactory mordant and Merck's pure technical acetone is an excellent differentiating agent. A system is established by means of the dyes and reagents which form a physico-chemical equilibrium, provided pure dyes are employed, and the technic is carried out with precision. Gram-positive bacteria are coated by means of buffered crystal violet solution and the iodine-sodium hydroxide solution precipitates the crystal violet from other substances. The dye-iodine precipitate is readily dissolved by pure acetone. Iodine green, a pure derivative of crystal violet has the effect of non-interference in the technic and has selective action upon nuclear substance. Pyronin has affinity for Neisserian organisms primarily and acts as an inert substance upon most other proteins, (except cytoplasm of eosinophiles, lymphocytes, plasma cells, and endothelial cells). The following technic is recommended:**

Stain air-dry films 3 to 5 minutes in a 1% solution of crystal violet in 10 parts of Clark and Lubs' phosphate buffer of pH 6.6 to 7.0 and 90 parts water. Decant and flush with 2% iodine in N/10 NaOH. Decant and decolorize in acetone 10 seconds or less. Air dry and counterstain 1½ to 2 minutes with methyl-green-pyronin (2 parts 2% aqueous methyl green National with one part 0.3% aqueous pyronin yellowish). Wash and air dry. Oil of Bergamot is preferable to xylene as a clearing agent. Best results are obtained if each slide is handled separately as for staining blood films.

Standardization of staining methods for the diagnosis of gonorrhea has been the subject of considerable controversy. Work in recent years with the alkaline method of Gram's technic, first employed by Jensen (1912) in Europe and later by Burke (1921) in this country,

[1]This study was initiated at the Willard Parker Hospital, New York City under the auspices of the Milbank Memorial Fund and was completed at the City Hospital. Acknowledgment is hereby given to Drs. John C. Torrey, Wm. H. Park and H. J. Conn, whose professional counsel made the work possible.

has led to better standardization. The recent papers by Churchman (1927, 1929) and by Burke and Barnes (1929) gave excellent proof of this. Approach to standardization of Gram's technic has been contemporaneous with increasing demand by physicians for the Pappenheim method of staining. The original method of Pappenheim, (1889), was devised as a differential cytological stain, and was later (1901) applied to examination of exudate for evidence of Neisserian infection. The Pappenheim-Saathof mixture (see Conn, 1929, p. 119) is now employed for this purpose, and of the latter the following excerpt from Hiss and Zinsser's text book (Zinsser, 1929, p. 461) gives resume: "An excellent picture is obtained with the Pappenheim-Saathof stain.....Gram's method, however, is the only one of differential value."

The Medical Committee of the Bellevue-Yorkville-Health Demonstration in charge of a venereal disease survey instructed me, in May 1929, to concentrate upon staining methods for the purpose of finding a pure dye which might be specific for the gonococcus. A question naturally occurred at that time: "Could the Gram method be so modified as to incorporate a cytological differentiation with the brilliant effect of the Pappenheim methyl green-pyronin mixtures?" Experimentation was begun which led to the manufacture (by the National Aniline and Chemical Company) of two dyes not previously prepared in this country. The first of these dyes is an over-ethylated methyl green similar to Grübler's iodine green (iodgrün), which is shortly to be put on the market in this country in place of the regular National Aniline methyl green.[2] The other dye is a pyronin of the type manufactured in Europe under the name of pyronin G (i. e., gelb), which will be put on the market in this country under the name pyronin yellowish. Previous pyronins manufactured in America have been of the type designated in Germany pyronin B (i. e., blau). One American company, Coleman and Bell, put on the market a pyronin of the yellowish type, but they state that it is not of American manufacture. These two new dyes were prepared by the National Aniline and Chemical Company with the coöperation of the Biological Stain Commission. A mixture of them has been employed as counterstain in a modified alkaline Gram's technic.

Technic for the Differential Gram Stain with New Dyes

Films are made from exudate which has been skilfully selected. The exudate is collected by means of sterile cotton swabs, which are

[2]All lots of methyl green, manufactured by this company, bearing certification numbers higher than NG7, are to be of this type. They will be labeled "methyl green National."

rolled (not rubbed or dragged) over clean new glass slides. The films are air dried, fixed by means of gentle heating and are stained in the following solutions. (Heat fixation gave slightly better results than fixation by means of alcohols.)

1. Flush the slide with buffered crystal violet solution 3 to 5 minutes.

   Crystal violet ..... 1 g.
   Distilled water ..... 90 cc.
   Buffer solution (pH 6.6 to 7.0) ..... 10 cc.

   The phosphate buffer is prepared according to the method of Clark and Lubs.
2. Decant and flush with iodine solution so that the dye-iodine precipitate overflows. Time 1½ to 2 minutes.

   Resublimed iodine ..... 2 g.
   Distilled water ..... 90 cc.
   N/1 NaOH ..... 10 cc.

   Solution of iodine in phosphate buffer was unsatisfactory.
3. Decant and decolorize by means of Merck's pure technical acetone, adding the acetone drop by drop until the drippings are colorless. Time 10 seconds or less. Air dry.
4. Counterstain with the following differential methyl-green-pyronin mixture 1½ to 2 minutes. Prepare counterstain a day or two before using. This counterstain can be employed in examination of formalized tissue also; (see Scudder and Lisa, 1931).

   Pyronin yellowish ..... 0.1 g.
   Over-ethylated methyl green[3] ..... 1.25g.
   Distilled water (hot) ..... 99. cc.

   Wash quickly with tap water. Air dry and examine by means of oil immersion lens and artificial daylight. Oil of bergamot is preferable to xylene for clearing the slides.

The Results

Gram-positive bacteria are purple black. The majority of Gram-negative bacteria are faded pink; however, a small influenzal bacillus which has frequently been encountered in exudate stains deep magenta. The capsule of Friedländer's bacillus stains a transparent lavender.

The polynuclear nucleus is dark blue-purple, the cytoplasm faint lavender. The lymphocyte nucleus is homogeneous very deep clear blue, the cytoplasm faint magenta violet to magenta. The plasma cell nucleus is dark navy blue with cart wheel arrangement, the cyto-

[3]See footnote 2.

plasm homogeneous deep magenta to purple-magenta. The eosinophile nucleus is like that of the polynuclear and granules are fairly distinct reddish and occasionally purplish. The endothelial nucleus is pale blue, the cytoplasm pinkish lavender. The red blood cell is pale magenta to bronze. Bronzing of the red blood cell is due to impregnation of dilute iodine. The nucleus of the squamous epithelial cell is purple, the cytoplasm lavender. Purplish tinting of the epithelium is due to retention of dilute crystal violet dye. The nucleus of the columnar epithelial cell of the urinary tract is more blue than that of other epithelium and the cytoplasm stains more deeply magenta. Retention of pure methyl green gives the blue tinting and retention of dilute pyronin gives the magenta tinting. The phagocytic endothelioid cell is more reddish and more finely granular than other cells and may be found packed with gonococci in the free exudate from cases of gonorrhea.

Comments and Summary

Initial experiments were made with emulsions of Gram-negative micrococci and sarcinae in egg albumen and ascitic fluid. The films were stained by means of Kopeloff and Beerman (1922) modified Gram technic and counterstained with mixtures of aqueous solutions of methyl green and pyronin. Two commercial dyes, a methyl green and a pyronin made a satisfactory differential counterstain for the alkaline method of Gram. Albuminous fluid stained pale lavender. Neisserian organisms stained deep magenta. Sarcinae stained deep purple in young culture and pale pink to magenta in old culture.

Differentiation of organisms in serous exudate was studied by means of inoculation experiments with guinea pigs. Young cultures[4] of gonococci, *Neisseria catarrhalis*, sarcinae, and the atypical strain No. 115 (Scudder, 1930) were introduced intraperitoneally, recovered with the peritoneal exudates within eighteen hours, spread in films, stained and examined. Strain No. 115, isolated from an infantile case of cervico-vaginitis, was morphologically like a gonococcus in intracellular arrangement and was fermentatively and agglutinatively like type III meningococci, but unlike meningococci by agglutinin absorption. Films of the exudate stained by means of the routine Gram and the differential counterstain showed Neisserian organisms bright red, sarcinae deep purple and serosal cells stained as in the Pappenheim stain. Partially phagocytized sarcinae were lavender or pale pink.

[4]Cultures isolated from cases of cervico-vaginitis and studied in the laboratories of the New York Dept. of Health.

This method of counterstaining was used routinely in the Gram's technic for examination of films from cases of infantile as well as adult gonorrhea and the following information was elicited. Certified methyl green of the old type (NG4) was most stable and the least interfering to the technic. Grübler's iodine green, recommended by Torrey and Buckell (1922) for use in differential medium for the gonococcus, gave results most like the former and appeared slightly more blue-green. Unknown to me, Pappenheim (1899) had made this statement: "Statt des Methylgrüns kann man auch Jodgrün aber nicht Malachitgrün odor Chromgrünanwenden." Pappenheim adhered to the routine use of methyl green dyes and there has been little demand for iodgrün in histology or bacteriology. Coleman and Bell's pyronin (CP1) and Grübler's pyronin were the best red dyes for the technic and an imported red dye, rhoduline red G, was a fair substitute for either of them. The two American dyes which were ultimately manufactured gave results which were as good as the best results obtainable with imported dyes and were pure and standard in quality. They proved satisfactory for the differential counterstain in the alkaline Gram's technic both for examination of free exudate and formalized tissue. (See Scudder and Lisa, 1931.) The findings for stained sections of tissue are published separately.

In the course of the search for a satisfactory methyl green, special samples of methyl green chloride, iodide, and bromide were prepared by the Color Laboratory of the Dept. of Agriculture.[5] With the bromide there was observed greatest intensity of the staining and penetration of nuclear substance by the green; with the chloride, the pyronin staining of the cytoplasm was most pronounced; while the iodide seemed to give a differentiation mid-way between the other two dye salts.

Two impurities in samples of methyl green became apparent: 1) the presence of zinc; and, 2) the presence of free crystal violet dye. The former caused uneven streaking of the films, the latter caused too much purpling of the film. Samples of methyl green which were practically free from crystal violet were clear blue-green in color.' Samples which contained considerable crystal violet were metallic purple-blue in color. Purity of the dyes in reference to low crystal violet content could be determined by placing a small quantity of the dye powder on a piece of filter paper, flecking distilled water over it and noting the quantity of purple streakings. Samples of methyl green which gave best results were practically 100% free from crystal violet.

[5]To Mr. W. C. Holmes of that laboratory acknowledgment for this assistance is hereby given.

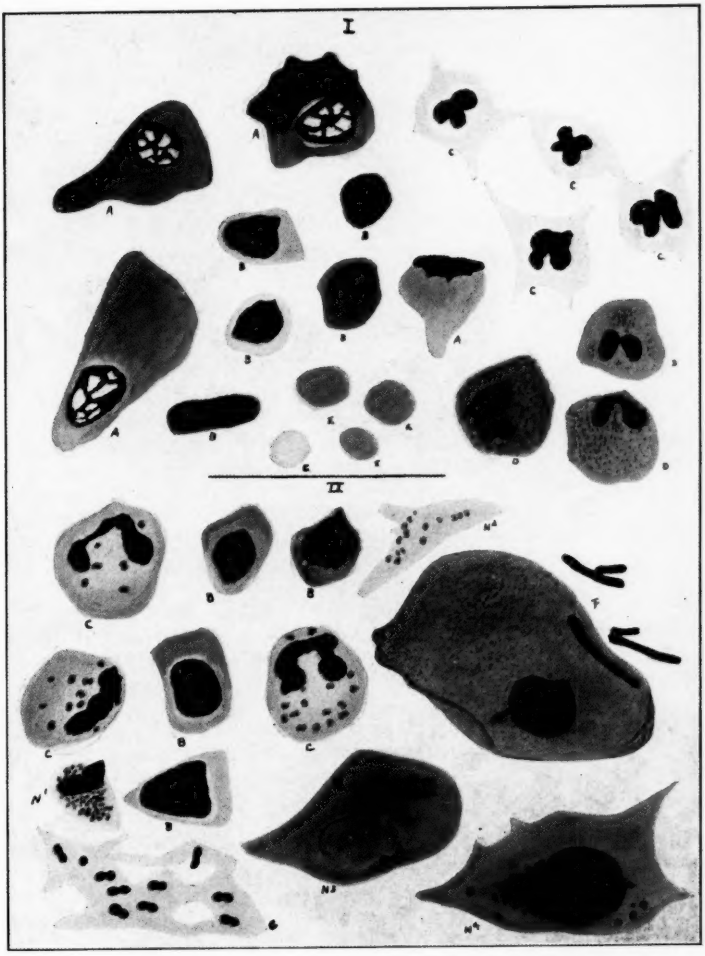
EXPLANATION OF COLOR PLATE

I. Tissue fixed in 10% formalin, embedded in paraffin and stained to show cytological differentiation.

II. Serous exudate stained to show cytological and bacteriological differentiation.

- A. Plasma cells
- B. Lymphocytes
- C. Polynuclear cells (containing gonococci in II).
- D. Eosinophile cells
- E. Red blood cells
- F. Squamous cells and Gram-positive bacilli
- G. Pneumococci
- N. Characteristic arrangement and morphology of gonococci.

1. In disintegrated phagocyte
2. Mucus
3. Endothelial cell (lining type of cell).
4. Monocyte.


I
A
B
C
D
E
F
II
N1
N2
N3
N4

The use of Sorensen's (Merck's anhydrous blue label) phosphate in solution of crystal violet for staining the Gram-positive bacteria was incorporated as a new feature. The solution remained homogenous for many weeks and months and disintegration proceeded most slowly on the acid side of neutrality. The Hucker (1922) ammonium oxalate-crystal violet solution also has the effect of a buffered solution and may be employed with the new differential counterstain. Attempts will be made to stabilize the mixture of dyes in the counterstain so that they may be successfully employed in very old mixtures. At present the mixture is prepared several hours before it is used and has given good differentiation upon standing four or five days.

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A NEW AND RAPID METHOD FOR MAKING PERMANENT ACETO-CARMIN SMEARS

WILLIAM CAMPBELL STEERE

Botanical Laboratory, University of Pennsylvania, Philadelphia, Pa.

ABSTRACT.—**Smear the pollen mother cells of a single anther from each flower bud on a clean dry slide, using a small scalpel. Flood the slide with Belling's aceto-carmin and heat for a second over an alcohol flame. Examine under the microscope to determine the stage of microsporogenesis. If the stage is satisfactory, smear the remaining anthers in the same manner, but fix and stain them by immediate immersion, face downward, in a petri dish full of hot (steaming) aceto-carmin for from 1 to 10 minutes. Then rapidly transfer thru the following mixtures: two parts 99% (glacial) acetic acid plus one part absolute ethyl alcohol; one part acetic acid plus two parts absolute alcohol; and finally one part acetic acid plus nine parts absolute alcohol. The slides are then to be dehydrated completely by 1 to 2 minutes immersion in pure absolute alcohol, and cleared 2 to 3 minutes in a mixture of xylene and absolute alcohol in equal parts. The preparations are then made permanent by mounting each with balsam and a cover glass. The whole process takes from 5 to 15 minutes and is particularly recommended for chromosome counts.**

For several years the author has been engaged in cytological and genetical investigations in which it frequently has been found necessary to determine the chromosome number of each of many individuals resulting from the crossing of forms with different chromosome numbers. The aceto-carmin smear method introduced to plant cytology by Belling (1921) has been found to be eminently suited to such undertakings, when the chromosome number is of incidental value only. If, however, as is usually the case, permanent records in the form of slides are wished, the most serious fault of this method appears. Since the aceto-carmin method, as described by Belling, gives essentially temporary results, even when the preparations are sealed, the author has heretofore been forced to rely upon much slower methods for his permanent slides.

Belling (1926) has casually mentioned making aceto-carmin mounts permanent by passing them thru grades of acetic acid into balsam. McClintock (1929) has improved upon Belling's suggested procedure and has given details for an efficient method of making aceto-carmin smears permanent. Her procedure depends, however, on macerating

the anthers in aceto-carmin, heating the preparation in order to affix the pollen mother cells to the slide and cover glass, and then soaking them apart with dilute acetic acid. Great care must be taken during the process not to dislodge the pollen mother cells, and both the slide and cover glass have to be carried thru the stages of glacial acetic acid, then dehydrated and finally reunited in balsam.

Considering the time and patience required for the above procedure, the permanent smear methods of Taylor (1924) and Mann (1924) and the various modifications of the former have been found more efficient, since they require about the same length of time and give results much finer and more accurate in all ways. Newton's (1927) modification of Taylor's smear method has been found particularly useful in the past.

In order to secure large numbers of chromosome counts in the shortest possible time, the author has devised a very simple and rapid method for making permanent aceto-carmin smears.

Flower buds from the plants to be investigated are brought into the laboratory from the field or greenhouse and kept fresh in moist paper until ready for use. One anther is removed from a bud of approximately the right size, and the pollen mother cells smeared out on a clean dry slide with a small scalpel, especially honed flat for this purpose. The anther fluid and disintegrating tapetal cells are viscous enough so that the sporocytes adhere rather firmly to the glass. The slide is then flooded with Belling's aceto-carmin (45% glacial acetic acid saturated with carmin,[1] then filtered) and heated over an alcohol lamp until the liquid steams, when it may be examined microscopically. With some practice, the stage of microsporogenesis may usually be determined immediately, using the low power, with which a cover glass is not necessary. If the proper stage is discovered, the remaining anthers are smeared and stained in the same fashion, otherwise the test slide is wiped clean and the bud discarded. If the pollen mother cells of the test slide tend to loosen up and float away when the aceto-carmin is poured on, or when the slide is heated, an alternative method is recommended. Immediately after smearing, instead of being flooded with aceto-carmin, the slides are immersed, smeared-side downward, in the cover of a petri dish filled with hot aceto-carmin. A very short piece of small glass rod or tubing cemented to the bottom of the dish, at one side, will serve to hold one end of the slide from the bottom and prevent the pollen mother cells from being

[1]Good results were obtained with pre-war Grübler's carmin, with Coleman and Bell's carmin and National Aniline carminic acid, but not with carmin Merck. Poor results are sometimes due to using insufficient dye to saturate the solution.

rubbed off. The cover of a 10 cm. petri dish allows room for two slides side by side. This latter method has been found to solve the difficulty in the frequent cases in which the sporocytes tend to loosen, since the liquid meets the surface of the slide with equal force at all points, and the heat apparently causes the cells to adhere more firmly. The heat of the fixative may be maintained by keeping the petri dish on a hot plate or substage microscope lamp.

The process of fixing and staining in the aceto-carmin will take from one to ten minutes, depending on the material. The time required for the species under investigation is soon determined, and the procedure can be standardized accordingly. If the chromosomes do not stain deeply enough within this time, a small amount of ferric hydroxide added to the stain will remedy the difficulty, as Belling first discovered. A few iron filings in the aceto-carmin will do just as well, or even the blade of a steel scalpel held in the staining solution on the slide.

When a satisfactory stain has been secured, the aceto-carmin is drained off as completely as possible and the slides rinsed carefully in a Coplin jar containing two parts 99% (glacial) acetic acid and one part absolute alcohol. This wash usually removes all anther walls and other debris. The slides should next be transferred rapidly thru a mixture of one part acetic acid and two parts absolute alcohol into one part acetic acid plus 9 parts absolute alcohol, where they may be left from thirty seconds to two minutes. A prolonged stay in the first two acetic acid-absolute alcohol mixtures is liable to remove the stain from the chromosomes as well as from the cytoplasm. Dehydration is completed by allowing the slides to remain 1 to 2 minutes in absolute alcohol. Clearing is accomplished by transferring the slides to a mixture of xylene and absolute alcohol in equal parts, leaving them for 2 to 3 minutes. A cover glass is then placed on the smear with a drop of balsam, and the permanent slide is complete.

The method of mounting in Canada balsam directly from the mixture of absolute alcohol and xylene, as suggested by McClintock, was found to be entirely satisfactory. It is also considerably more rapid than running the slides thru the several stages of absolute alcohol and xylene which would be required to bring the fresh microsporocytes into pure xylene without undue shrinkage.

The chromosomes in successful preparations will appear from dark red to deep purple, while the cytoplasm will be practically colorless. The slightly acid reaction of Canada balsam and the small amount of acid carried over in the cells should tend to keep the stain from fading. With care, a counterstain may be added in the absolute alcohol stage.

If the cytoplasm appears very dark, a shorter staining period is recommended. Overstained slides may be differentiated singly by flooding them with pure glacial acetic and warming moderately, until the stain has reached the desired intensity. In routine work on material which persistently overstains, a well of glacial acetic may be interpolated between the stain and the first acetic acid-absolute alcohol mixture. It should also be kept in mind that heat applied during the staining process will intensify the stain in the chromosomes and usually keep the cytoplasm from staining too heavily. This effect of heightened temperature on the stain's selectivity for chromatin has been discussed previously by McClintock.

The following schedule is included for reference:

1. Secure fresh flower buds of the desired plant.
2. Smear the pollen mother cells from one anther on a dry slide.
3. Immediately flood the smear with aceto-carmin.
4. Heat carefully over an alcohol flame until the stain steams.
5. Examine under the low power of the microscope (a cover glass is not necessary) after 1 to 5 minutes of staining.
6. Smear remaining anthers if the stage is satisfactory.
7. Stain these slides by either flooding with, or immersion in, aceto-carmin, for 1 to 10 minutes.
8. Two parts glacial acetic acid plus one part absolute ethyl alcohol. Rinse.
9. One part glacial acetic acid plus two parts absolute ethyl alcohol. Rinse.
10. One part glacial acetic acid plus nine parts absolute ethyl alcohol. ½ to 2 minutes.
11. Equal parts xylene and absolute ethyl alcohol. 2 to 3 minutes.
12. Mount in Canada balsam.

During the winter months of 1930–31, this method was tried out on several different species, both monocotyledons and dicotyledons. Good results were secured from *Rhoeo*, *Tradescantia* and *Velthemia*, while *Nicotiana* and *Petunia* gave entirely satisfactory results. The method is now being tried on animal material.

The procedure, as outlined above, is recommended for securing chromosome counts in pollen mother cells, as is often necessary in genetical work. It may also be used for more detailed cytological work, especially if the smears are made upon cover slips, which are then treated in the same manner as slides. This method brings the pollen mother cells into direct optical contact with the lens, thru the medium of the immersion oil.

The rapidity and simplicity of this method is due mainly to the fact that the anthers are smeared on the slide before the aceto-carmin is added, instead of being macerated into a drop of the stain. In the latter case, it is rather difficult and tedious to secure a good permanent mount without losing many of the sporocytes.

When working with a plant which responds well to this method, permanent slides, ready for examination under the lower powers of the microscope, may be prepared in from five to fifteen minutes, since a satisfactory stain may usually be secured in from two to three minutes, and only a minute or so is required in each grade of dehydrating and clearing solution. If material is abundant, a series of slides may be kept going thru the various grades, in succession, so that it is often possible to make a large number of permanent preparations in a short time.

This technic, then, appears to be one of the most rapid yet evolved for the preparation of permanent slides demonstrating meiosis in pollen mother cells.

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